Review

Approaches and challenges to engineering seed phytate and total phosphorus

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ABSTRACT

About 75% of seed total phosphorus (P) is found in a single compound, phytic acid (myo-inositol-1,2,3,4,5,6-hexakisphosphate or $InsP_6$). Phytic acid is not efficiently utilized by monogastric animals (poultry, swine, fish), which creates phosphorus management and environmental impact problems in animal production. Phytic acid also functions as an antinutrient when consumed in human and animal diets. These problems can be addressed via feed or food supplementation with P and other minerals or phytase, or more efficiently and sustainably at their source by crop breeding or bioengineering of low-phytic acid/high-available P crops. However, since phytic acid and its synthetic pathways are central to a number of metabolic, developmental and signaling pathways important to plant function and productivity, low-phytate can translate into low-yield or stress susceptibility. The biological functions of phytic acid and identification of genetic resources and strategies useful in engineering high-yielding, stress-tolerant low-phytate germplasm will be reviewed here. One promising approach that can avoid undesirable outcomes due to impacts on phytic acid metabolism is to engineer "high-phytase" seeds. In contrast to the issue of seed phytic acid, there has been relatively little interest in seed total P as a trait of agricultural importance. However, seed total P is very important to the long-term goal of sustainable and environmentally friendly agricultural production. Certain low-phytate genotypes are also "low-total P", which might represent the ideal seed P trait for nearly all end-uses, including uses in ruminant and non-ruminant feeds and in biofuels production. Future research directions will include screening for additional genetic resources such as seed total P mutants.

Published by Elsevier Ireland Ltd.

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1. Introduction

Developing seeds accumulate more phosphorus (P) than needed for nominal cellular function. Higher plants synthesize this excess P into phytic acid (*myo*-inositol-1,2,3,4,5,6-hexakisphosphate or InsP$_6$; Fig. 1A [1,2]) and subsequently deposit it as mixed salts of macro- and micromineral nutrients, referred to as phytins [3]. These mixed salts primarily contain K and Mg, and to a lesser extent Ca, Fe and Zn and other minerals, and are often deposited as discrete inclusions (globoids) in one class of the storage microvacuoles referred to as protein storage vacuoles (PSVs). Phytic acid is ubiquitous in eukaryotic cells. It is the most abundant inositol phosphate in nature and is a key component of numerous developmental and signaling processes. These occur in the cyotoplasm and plastids, such as P and mineral storage and signal transduction that involves transient calcium flux, and in the nucleus in pathways important to DNA repair, chromatin remodeling, RNA editing and the regulation of gene expression [4–7]. Since a substantial fraction of P taken up into vegetative plant tissues is subsequently translocated to developing seeds, and since 75% ($\pm$10%) of this P is synthesized into phytic acid, it turns out that seed phytic acid P represents a significant bottleneck in the flux of P through the world's agricultural ecology. The net phytic acid P stored in the seed of the major crops is equal to a sum equivalent to greater than 65% of the P applied annually as fertilizer, worldwide [8].

Phytic acid is an efficient chelator of nutritionally important positively charged mineral cations and forms stable salts of Ca, Fe and Zn in the nutritional tract [9]. Most of the P and minerals in these salts are not utilized. They are excreted, potentially contributing to nutritional deficiency in human populations [10,11]. There probably are also health-beneficial roles for dietary phytic acid in humans [12–17]. However, clinical evidence for these beneficial roles is lacking, in contrast to the abundant evidence for the negative roles of phytic acid in both human nutrition and in P utilization and management in animal agriculture [2,18]. Phytic acid P is non-available for monogastric animals (Fig. 1B [19]). The 20–30% of seed total P that is non-phytic acid P is available P, but is not sufficient for optimal animal productivity. To provide optimal levels of P for animal productivity, historically feeds were supplemented with a nutritionally available form of P, such as dicalcium phosphate. However, while P supplementation provides sufficient P for animal productivity, it does not reduce excretion of phytic acid derived-P, an environmental hazard and a waste management problem [20]. One effective approach to this problem is to supplement feeds with the industrially produced enzyme phytase [19]. Following consumption, phytase activity breaks down phytic acid, releasing its phosphate for utilization by the animal. Thus a greater portion of the animal's need for P is supplied by the grain component of the feed, and less is excreted.

Approaches to problems represented by seed phytic acid include engineering crops to express high levels of phytase enzyme in seeds [19], or breeding crops with reduced levels of seed phytic acid (low-phytate or high-available P [2,18]). Nearly 20 years ago the first *low-phytic acid* (*lpa*) genotypes of crop species were isolated in maize (*Zea mays* L.), using chemical mutagenesis and classical genetics [21]. Today there are numerous *lpa* genotypes of maize, wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L.), barley (*Hordeum vulgare* L.), soybean (*Glycine max* (L.) Merr.), *Arabidopsis thaliana* L. and most recently common bean (*Phaseolus vulgaris* L. [2,22,23]). The molecular genetics of seed phytic acid metabolism and phytase enzymes has also advanced, adding genetic engineering tools to approach this problem area [24,25].

In low-phytate crops, phytic acid P reductions are often matched by molar-equivalent (in terms of P) increases in seed inorganic P, so that seed total P is largely unchanged (Fig. 1B). As a result, available P (in terms of human and animal nutrition) is greatly increased. Numerous animal nutrition studies with poultry, swine and fish species have shown that, depending on feed formulation, the use of low-phytate types of maize, barley, and soybean can result in increased feed P utilization and reduced waste P, in proportion to the decrease in phytate P and increase in non-phytate P in the low-phytate grain or legume [2,18]. Human nutrition studies have documented 30% to 50% increases in "fractional absorption" of Fe, Zn and Ca, in subjects consuming low-phytate maize types versus normal-phytate types [26–28]. An alternative plant breeding approach to dealing with mineral deficiency in human populations is to breed for increased mineral content (biofortification [29]). It turns out that the range of increases in mineral bioavailability achieved via seed phytate reduction in *lpa* crops, 30–50%, could contribute substantially to achieving the targets set for enhanced seed mineral content and bioavailability established for the HarvestPlus project [30].

The genetic resources represented by *lpa* mutations and alleles has contributed to a number of studies that have identified functions important to phytic acid and its metabolic pathways and to plant and seed performance. These studies have often revealed negative impacts on yield and plant performance resulting from perturbation in phytic acid metabolism. In addition, recent studies indicate a high level of integration of metabolic and signaling pathways that involve inositol phosphates and phytic acid. As a result, engineering seed phytate levels while maintaining plant and seed function, quality and yield could prove challenging. The
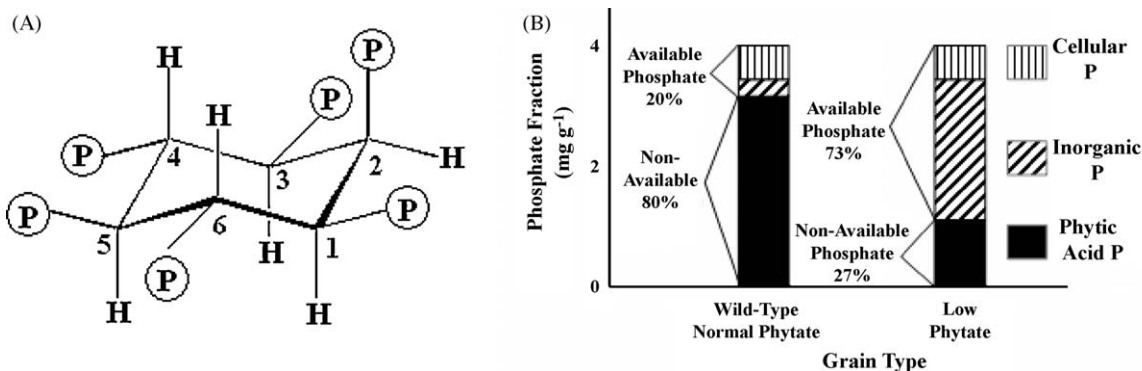
Fig. 1. (A) Chemical structure of phytic acid (*myo*-inositol-1,2,3,4,5,6-hexakisphosphate or InsP$_6$). Numbers refer to the carbon atoms in the *myo*-inositol (Ins) backbone, and follow the "D-numbering" convention. "P" = H_2PO_4. (B) Seed phosphate fractions in standard "wild-type" grains and legumes and in an example of low-phytate lines or cultivars. "Cellular P" represents the sum of all P-containing compounds other than phytic acid P and inorganic P, such as DNA, RNA, proteins, lipids and carbohydrates. "Available" and "non-available" phosphate refers to nutritional availability for non-ruminants such as poultry, swine and fish, and is based on the assumption that "available P" for non-ruminants approximately equals non-phytic acid P, the sum of all P other than phytic acid P.

effort to navigate through the important biological functions of phytic acid to identify genetic resources and strategies useful in engineering high-yielding low-phytate germplasm will be reviewed here.

Seed total P is very important to the long-term goal of sustainable and environmentally friendly agricultural production. Both increased or decreased seed total P, as compared with standard types, would have value for specific end-uses. There is an ongoing effort to use plant product-based feeds as substitutes for fish meal in aquaculture feeds [31], but such products have less than adequate total P for optimal production [32]. Increased seed total P might also be beneficial for seed function and performance [33]. However, the trend in both ruminant and non-ruminant animal agriculture research is to maximize efficiency and minimize waste in part by identifying the minimal level of nutrient inputs, including feed P, necessary to maintain production [32,34–37]. In the case of ruminant (dairy and beef) production, grain and legume products probably contain ∼25% more total P than cattle require [37–39]. The excess feed P ends up in the manure and represents a significant waste management problem. Reduced total P might also be a desirable trait for grains used in biofuel production. Increased use of grains for biofuel production has increased the supplies of milling byproducts such as distillers dried grains (DDGs). DDGs contain 2- to 3-fold higher total P than do whole grains, and increased use of such byproducts in feeds exacerbates the waste P management problem [37].

Perhaps the most serious long-term issue relating to seed P is that the world supply of rock phosphorus readily available for mining and production into fertilizer or feed P might prove limiting to food production within 50 years [40–42]. Since seed total P represents a major bottleneck in the flux of P through the agricultural ecology, seed total P might prove a valuable target for reducing the need for P in agricultural production. There may be a potentially useful interaction in the biology of seed phytic acid P and seed total P; at least one low-phytate genotypes is also "low-total P" [18]. This probably represents the ideal seed P trait for nearly all end-uses, including use in ruminant and non-ruminant feeds or in biofuels production. The intersection of the genetics of seed P composition and total amount are also reviewed.

2. Gene targets for engineering the low-phytate trait

2.1. Inositol and Ins P_1 synthesis, Target Area 1

The biosynthesis of phytic acid requires the supply of its carbon backbone, the 6-carbon cyclic alcohol myo-inositol (Ins; Fig. 2, top center). The sole source of the Ins ring is via the conversion of glucose 6-P to a specific Ins monophosphate, Ins(3)P_1, which contains a single P ester at the "3" position [43]. This reaction is catalyzed by myo-inositol-3-P_1 synthase (MIPS). Plant genomes may contain from one (barley) to several (maize and soybean) copies of MIPS-encoding genes [44–46]. In genomes with multiple copies, the expression of one copy may be specific to the supply of Ins for phytic acid synthesis in the seed [46,47]. Also, MIPS expression may be targeted to the site of phytic acid synthesis within the cells and tissues of developing seeds [48]. Since MIPS activity is the sole source of the Ins ring, and since Ins is an essential cellular metabolite important to numerous pathways and functions (Fig. 3), perturbing MIPS expression may prove deleterious if not lethal, even if a seed-specific MIPS is targeted [49]. However, a recent study indicated that seed-tissue targeted partial down-regulation of MIPS results in the desired low-phytate phenotype with minimal undesirable effects on plant and seed function [50]. The pathway to phytic acid probably first requires the hydrolysis of Ins(3)P_1 to Ins and inorganic P, via the action of Ins monophosphatase.

2.2. Conversion of inositol or Ins P_1 to phytic acid, Target Area 2

Ins serves as the initial substrate for one or both of two subsequent pathways to phytic acid, the lipid-dependent (Fig. 2, right) and lipid-independent (Fig. 2, left) pathways [1,22]. Essentially these two pathways only differ in their early intermediate steps leading from Ins to Ins trisphosphates. In most eukaryotic cells including plant vegetative tissues, the lipid-dependent pathway represents the main route to phytic acid and other Ins phosphates that are central to signal transduction, such as Ins(1,4,5)P_3 [4,22,51–53]. However, it may not be the major route to phytic acid in seeds (see below).

In the lipid-dependent pathway (Fig. 2, top right) Ins is first transformed into phosphatitdylinositol (PtdIns), a lipid with Ins as its headgroup. The Ins headgroup of PtdIns is then phosphorylated to yield PtdIns(4,5)P_2, a compound subsequently hydrolyzed via the action of a specific phospholipase C to yield Ins(1,4,5)P_3. Ins(1,4,5)P_3 is then further phosphorylated to yield phytic acid, probably via the action of three types of Ins polyphosphate kinases which phosphorylate the indicated positions on the Ins ring: 5-/6-kinases, also referred to as Ins trisphosphate (ITP) kinases; 3-/6-kinases, also referred to as Ins phosphate multikinases; and Ins polyphosphate 2-kinases. These kinases can act on other substrates, have multiple functions, and are referred to in the literature with a variety of names other than those used here for brevity. Their importance to phytic acid synthesis in seeds is illustrated by the fact that maize lpa2 encodes a 5-/6-kinase [54], and that mutations in the Arabidopsis Ipk2β and Ipk1genes, which encode a 3-6-kinase and a 2-kinase, respectively, also result in the low-phytate seed phenotype [22].

In the lipid-independent pathway (Fig. 2, top left), Ins is sequentially phosphorylated to phytic acid via a series of soluble Ins phosphates perhaps as follows: Ins → Ins(3)P_1 → Ins(3,4)P_2 → Ins(3,4,6)P_3 → Ins(1,3,4,6)P_4 → Ins(1,3,4,5,6)P_5 → Ins(1,2,3,4,5,6)P_6 [55,56]. In vivo, the actual Ins phosphate isomers in any given step may not be exactly as shown. The lipid-independent pathway might only differ from the lipid-dependent pathway in the Ins → Ins P_1 → Ins P_2 steps, catalyzed by Ins kinase (Ins → Ins P_1) and an Ins monophosphate kinase (Ins P_1 → Ins P_2). Genes encoding Ins kinase have been documented in maize [57] and rice [58], but at present there are no reports of Ins monophosphate kinase or of a gene encoding it. This activity is the missing link in these pathways. The subsequent phosphorylation to Ins trisphosphates such as Ins(3,4,6)P_3 and on to phytic acid probably uses the same suite of Ins polyphosphate kinases mentioned above. Importantly, the early steps of the "lipid-independent" pathway, catalyzed by Ins and Ins monophosphate kinases, may be truly specialized for phytic acid synthesis in seeds and other tissues that store P as phytic acid. Therefore, as discussed below, genes encoding these two types of enzymes might prove to be the best targets for engineering reduced seed phytate levels without impacting pathways and processes important to other functions in the plant and seed.

2.3. Tissue and/or intracellular compartmentation, transport and storage, Target Area 3

Several components of the phytic acid synthesis pathways probably are sequestered in different tissues or via intracellular compartmentation (Fig. 4, top and middle). The lipid-independent pathway ultimately involves the breakdown and re-synthesis of Ins(3)P_1, potentially a futile pathway (Fig. 4, top). This probably is an outcome of tissue and/or intracellular separation of Ins and phytic acid synthesis (Fig. 4, top); Ins synthesis by the Glu6P → Ins(3)P_1 → Ins pathway is part of general housekeeping and probably occurs in a tissue or cellular compartment separate from that of the phytic acid synthesis [59].
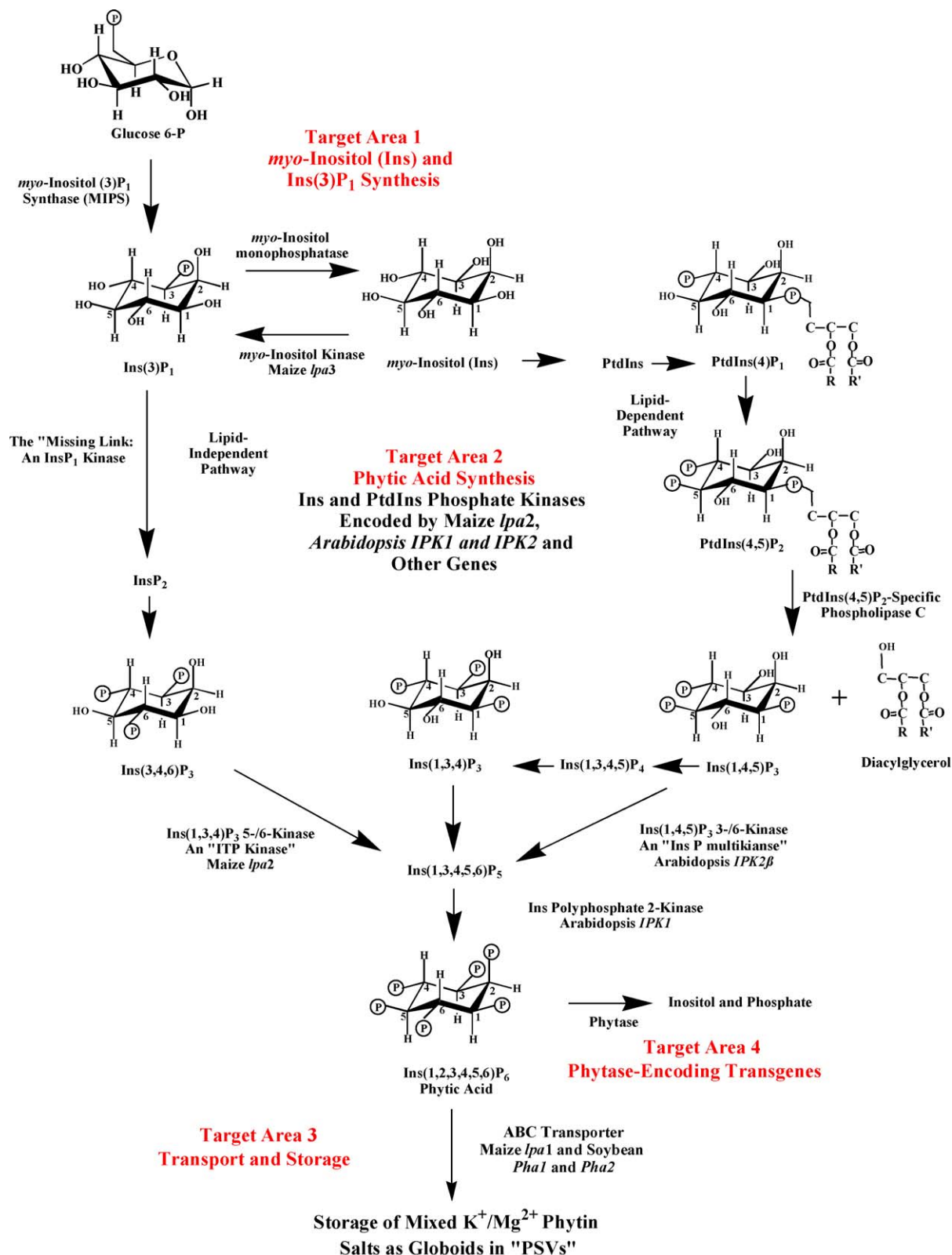
Fig. 2. Gene targets in the phytic acid biochemical pathways useful for engineering the low-phytate trait. Targets fall into four areas. *Target Area 1*: Inositol (Ins) and Ins(3)P$_1$ synthesis, starting with the conversion of glucose 6-P (top left) to Ins(3)P$_1$ (top left and top center). *Target Area 2*: Phytic acid synthesis. The "lipid-independent" pathway (center left) proceeds via sequential phosphorylation of Ins and soluble Ins phosphates. The "lipid-dependent" pathway uses precursors that include phosphatidylinositol (PtdIns) and PtdIns phosphates. *Target Area 3*: Transport and storage of phytin salts in globoids. *Target Area 4*: Phytase-encoding transgenes. The numbering of the carbons in the inositol ring follows the "D-numbering" convention. P = H$_2$PO$_4$.

Fig. 3. Pathways in plant biology that utilize *myo*-inositol (Ins).

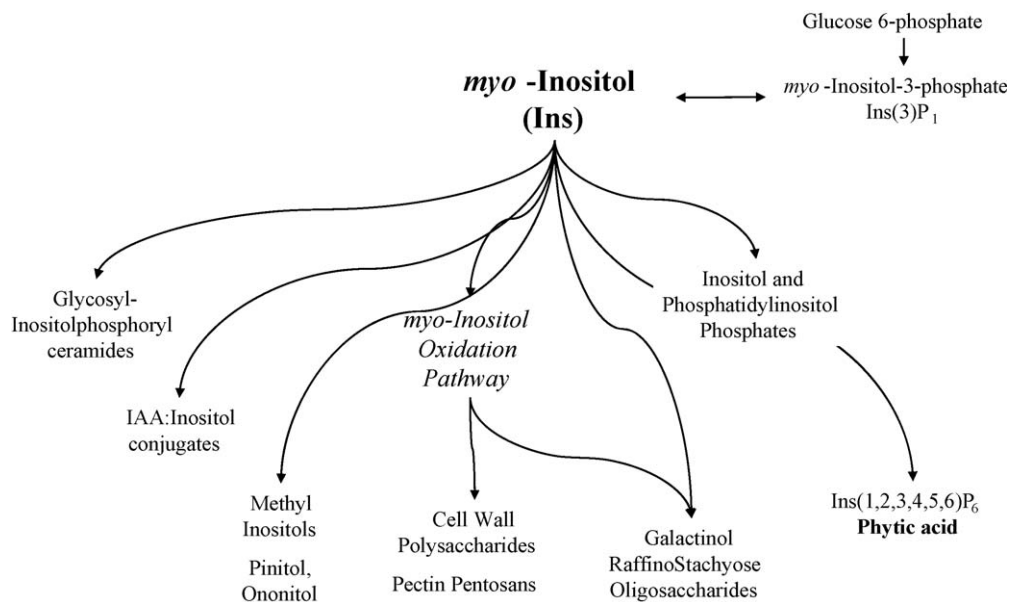A central issue in Ins phosphate metabolism is the cellular localization of Ins phosphate pools (Fig. 4). There are at a minimum two major cellular pools of inositol phosphates, nuclear and cytoplasmic. While inositol phosphates are soluble and rapidly diffuse within a compartment, the nuclear membrane represents a diffusional barrier [60]. Thus cytoplasmic and nuclear pools are synthesized by largely separate sets of proteins, and individual proteins may be targeted to the cytoplasm, or the nucleus, or to both compartments [61–63]. The cytoplasmic pathways probably contributes most to net seed phytic acid synthesis since cytoplasmic-targeted overexpression of a phytase active during seed development essentially abolishes seed phytic acid accumulation [24]. Therefore any evaluation of the biological role of a given protein important to these pathways, or its use in crop engineering, must consider its cellular localization. A second consideration is that plant genomes often contain multiple copies of genes encoding given steps in these pathways, but in some cases the relative contribution of each copy is not well characterized. A good example are the multiple copies of the Ins(1,3,4)P_3 5-/6-kinases (ITPKs) found in plant genomes [64]. Maize *lpa2* encodes such an enzyme [54] but the contribution of the other 5-/6-kinase-encoding genes in the maize genome is not known. More work needs to address the relative contributions of the lipid-independent and lipid-dependent pathways, and their distribution between cytoplasm and nucleus, to net phytic acid synthesis in seeds.

Following synthesis, phytic acid can serve as a counter ion for heavy metals, preventing toxicity. An example of this is the transient deposition/remobilization of Zn/Mn phytins during endosperm synthesis and remobilization in developing *Arabidopsis* seeds [65]. The relatively longer term storage of P, calcium, potassium, magnesium in mature seeds, in the germ and cotyledons of dicots or the germ and aleurone layer of monocots, is accomplished via transport of phytic acid and the mineral cations into protein storage vacuoles (PSVs) followed by deposition as mixed phytin salts (Fig. 4, bottom [3]). Therefore transport functions play important but as yet not well defined roles in both phytic acid synthesis and phytin storage.

Maize *lpa1* was the first gene in any eukaryotic species shown to encode a transport function important to phytic acid synthesis or accumulation. It encodes a multi-drug resistance-associated protein (MRP), one member of the ATP-Binding Casette (ABC) transporter gene family [25]. The exact role of this ABC transporter is not yet known but its importance to net seed phytic acid accumulation and seed viability is illustrated by subsequent findings. The low-phytate phenotype of the soybean M153 mutant [66] is due to co-inheritance of recessive alleles of duplicate, non-linked homologs of the maize ABC transporter [67] and the rice *XS-lpa2* mutant is due to a mutation in the rice homolog of the maize ABC transporter [68]. An at least partially functional copy of this ABC transporter is critical to seed viability, and possibly to plant viability. As homozygotes, null alleles of the maize *lpa1* and rice *lpa2* homologs both abolish phytic acid accumulation and are lethal, whereas intermediate or hypomorphic alleles are viable [68,69].

Several lines of evidence indicate that the maize *lpa1* locus is highly mutable. A practical outcome of this is that desirable phenotypes might prove unstable, and if this instability leads to a null allele, it would be lethal. In the initial screen of a chemically mutagenized population for maize *lpa* mutations, recessive alleles of maize *lpa1* were isolated at the rate of 1 per ∼200 M_2 progeny, a rate nearly an order of magnitude greater than a typical rate, and about 1 in 10 were lethal [69] (V. Raboy, unpublished data). It was subsequently demonstrated that paramutation occurs with maize *lpa1* alleles [70]. In the nucleus of a heterozygote containing a paramutagenic recessive *lpa1* allele and a paramutable wild-type allele, the wild-type allele can be altered via an epigenetic mechanism to an intermediate recessive allele, resulting in heritable and relatively stable reductions in seed phytic acid levels. In fact, this phenomenon occurs spontaneously at a relatively high rate, and may explain in part the high rate of mutation initially observed for the maize *lpa1* locus.

Epigenetic phenomena such as imprinting and paramutation are often affected though DNA methylation, and represent heritable events that impact gene expression but that are not due to changes in DNA sequence [71]. Epigenetic regulation is critical to normal development. It contributes to heritable changes in the regulation of gene function and expression, often on a genomic scale. This in turn is important to many processes and cellular functions. Paramutation was first discovered and mostly observed in studies of loci in the maize anthocyanin pathway, for which allelic variation is easily visually scorable [72]. An
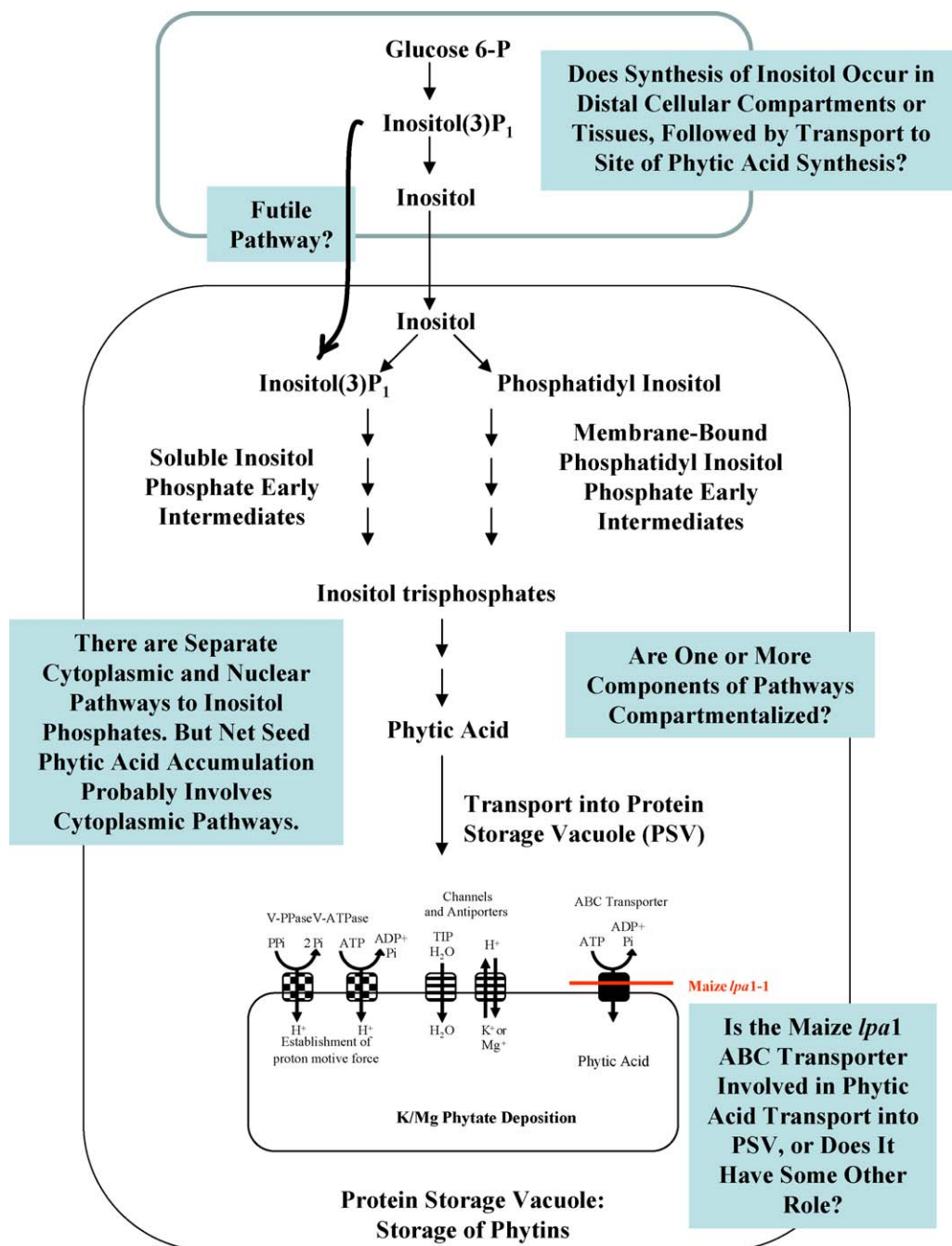
Fig. 4. Questions concerning cellular localization and compartmentazation of *myo*-inositol (Ins) and phytic acid synthesis and storage during seed development. V-PPase: vacuolar pyrophosphatase; V-ATPase: vacuolar ATPase; TIP: tonoplast intrinsic protein; ABC transporter: ATP-binding cassette transporter; PSV: protein storage vacuole.

outstanding question is if it is a rare phenomenon or alternatively impacts many genes and is therefore of broad importance to biological function [72]. Maize *lpa*1 is the first locus encoding a function critical to central metabolic pathways in which para-mutation occurs, possibly indicating that this phenomenon might be broadly important and might impact many genes and processes [70].

2.4. Rice lpa1: forward genetics continues to yield new knowledge

Following the map-based cloning of rice *lpa*1-1, database searches found homology to only one previously reported gene with annotated function, an archael 2-PGA kinase, which produces 2,3-bis-PGA [73]. While the function of the rice *lpa*1 protein has not

yet been unequivocally determined, there is evidence for a link between 2,3-bis-PGA and both Ins and Ins phosphate metabolism. The first evidence comes from studies addressing Ins metabolism in the slime mold *Dictyostelium* [74]. Ins synthesis mutants were generated and high levels of 2,3-bis-PGA were observed in the resulting Ins-depleted cells. 2,3-bis-PGA is an effective competitive inhibitor of Ins and PtdIns polyphosphate 5-phosphatases [75]. These enzymes recognize the neighboring ("vicinal") P esters at the "4" and "5" positions of Ins(1,4,5)P$_3$ or PtdIns(4,5)P$_2$ and remove the "5-position" phosphate. One of their functions is in signal termination: the breakdown of the second messenger Ins(1,4,5)P$_3$. The structure of the neighboring P esters of 2,3-bis-PGA mimics those in Ins(1,4,5)P$_3$ and thus 2,3-bis-PGA can occupy the active site of the 5-phosphatase, explaining the competitive inhibition.

The 5-phosphatase also displayed a low level of activity against 2,3-bis-PGA [75]. A further link between 2,3-bis-PGA, glycolysis and Ins phosphate metabolism, was elucidated via a phosphatase that acts both on Ins phosphates and glyceric acid phosphates. A mammalian enzyme similar to phytase, the "multiple inositol polyphosphate phosphatase" (MIPP), can also act on 2,3-bis-PGA to generate 2-PGA [76].

It was hypothesized that following Ins depletion in *Dictyostelium*, the synthesis of 2,3-bis-PGA may stabilize cellular levels of Ins phosphates important to cellular signaling such as Ins(1,4,5)P$_3$ [74]. A similar function for 2,3-bis-PGA might be important to phytic acid synthesis and accumulation in developing seeds. If currently underway studies demonstrate that rice *lpa*1 encodes an active 2-PGA kinase which produces 2,3-bis-PGA, its role in phytic acid synthesis might be analogous to its proposed role in *Dictyostelium*; to protect Ins polyphosphate intermediates from breakdown catalyzed by 5-phosphatases.

Another interesting possibility is that the same protein might also function as an Ins(3)P$_1$ kinase (Fig. 5A). This hypothesis is suggested by the underlying structural similarity of both the substrates 2-PGA and Ins(3)P$_1$ and the products 2,3-bis-PGA and Ins(3,4)P$_2$. As discussed earlier, the one key step in the lipid-independent pathway to phytic acid that has not been demonstrated either at the enzymatic or genetic levels, the "missing link", is the Ins(3)P$_1 \rightarrow$ InsP$_2$ step catalyzed by a putative Ins(3)P$_1$ kinase. The protein encoded by rice *lpa*1 might function both in the production of an important intermediate in phytic acid synthesis, and in the production of a second compound that protects intermediates to phytic acid from breakdown.

2.5. Phytase-encoding transgenes, Target Area 4

A great deal of progress has been made in the molecular biology and molecular engineering of microbial and plant phytases [77–81]. Phosphatases that display phytase activity to date fall into four classes: histidine acid phosphatases; β-propeller phytases; cystein phosphatases; purple acid phosphatases. The most recent novel contribution to the study of phytases was the discovery that a widely conserved eukaryotic gene first identified as the yeast (*Saccharomyces cerevisiae*) VIP gene contains both Ins phosphate kinase and phytase (histidine acid phosphatase) domains [80] (see Section 5.2). The protein(s) encoded by VIP genes therefore may have both kinase and phosphatase activities working together to integrate Ins phosphate metabolism. While progress in studies of plant phytases continues [81], most crop engineering work has utilized microbial phytases (see Section 4).

3. The challenge of breeding low-phytate crops: downstream impacts

The pathways to phytic acid involve three compounds or classes of compounds that represent metabolic pools central to cellular metabolism, development and signaling: Ins, phosphate and Ins phosphates. By impacting these cellular pools, perturbations of phytic acid synthesis have many downstream impacts. As previously mentioned in the cases of MIPS and ABC transporter mutants, they can alter plant or seed metabolism and chemistry in ways that impact viability. They can also impact end-use or nutritional quality, germination and emergence, disease susceptibility, and signal transduction important to stress response. Several of these downstream effects may result in reduced plant performance and yield. Targeted engineering of the low-phytate trait might avoid some or all of these negative impacts. Additional examples will further illustrate these downstream effects.

3.1. Seed P fractions: P homeostasis versus P storage

The most obvious change in the chemistry of *lpa* seeds is the several-fold increase in seed inorganic P (Fig. 1B). Sequestration of P during seed development is necessary for maintenance of normal cytoplasmic inorganic P levels (P homeostasis), in turn necessary for normal starch, protein and oil synthesis and accumulation. For example, the rate limiting step in starch synthesis, the major component of the maize grain, is catalyzed by the enzyme ADP-glucose pyrophosphorylase, and this enzyme is allosterically inhibited by inorganic P [82]. Therefore large increases in inorganic P might greatly impact these metabolic pathways and possibly be toxic to the developing seed's cells. But the isolation of viable *lpa* mutants in several species, each with "high-inorganic P" seeds and relatively good yields, indicates that this is clearly not the case. Chemical and histological studies of *lpa* seeds, including two recent metabolite profiling studies, indicate only relatively minor changes in seed structure and morphology, starch, protein or oil composition, and total P or P and mineral distribution [2,21–25,83–88]. Apparently inorganic P is sequestered in storage microbodies almost as efficiently as phytic acid and P homeostasis is largely intact in *lpa* seeds, minimizing many potential downstream impacts. However P storage, defined as the ability of mature seeds to retain P during the seed's resting stage and through subsequent imbibition and germination, might be perturbed in *lpa* seeds. Inorganic P leaches more readily from *lpa* seeds than it does P from seeds (V. Raboy, unpublished results). This perhaps could cause problems in pathogen or contaminant growth, especially if seed gets wet prior to planting.

While large changes in seed chemistry have not been observed in most *lpa* genotypes, relatively small changes in major seed constituents such as starch content or composition can have important effects on yield or plant and seed function. Dry weight reductions of about 10% are observed in seeds homozygous for maize *lpa* alleles as compared with sibling wild-type seeds maturing on the same ears [21]. Thus this dry weight loss is a seed-specific trait independent of the parental genotype. This seed dry weight reduction is primarily due to reduced starch accumulation, and can account for a significant fraction of the reduced yields observed for these *lpa* lines. *lpa* mutations also alter seed sucrose levels [88] and have as yet undefined impacts on other seed carbohydrate and P-containing fractions [89,90].

3.2. lpa isolines and yield studies

The first *lpa* lines used to study yield and plant performance of a low-phytate crop type were near-isogenic maize hybrid pairs either homozygous wild-type or homozygous *lpa*1-1, obtained following four generations of backcrossing [91]. Field trials indicated that overall, a yield reduction of about 6% was observed in the *lpa*1-1 hybrids as compared with the normal-phytate hybrids. Large differences were not observed in other aspects of plant growth and seed function, such as "cold-test" germination and flowering date. However, observations of more fully isogenic maize *lpa*1-1 and *lap*2-1 inbred lines (unpublished observations of lines obtained following six crosses to recurrent parents, V. Raboy) indicate clear delays in flowering date and large effects on germination and stress tolerance, as compared with "normal-phytate" siblings. These effects were probably not observed in the initial study because the lines that were evaluated, while on average 94% similar in genetic background, perhaps were still not nearly isogenic enough to reveal such performance differences.

Four barley *lpa* mutations that are downstream of Ins synthesis, and therefore probably are low-phytate due to perturbed Ins phosphate metabolism, reduce crop yield to a varying extent roughly proportional to their reductions in seed phytic [92]. The
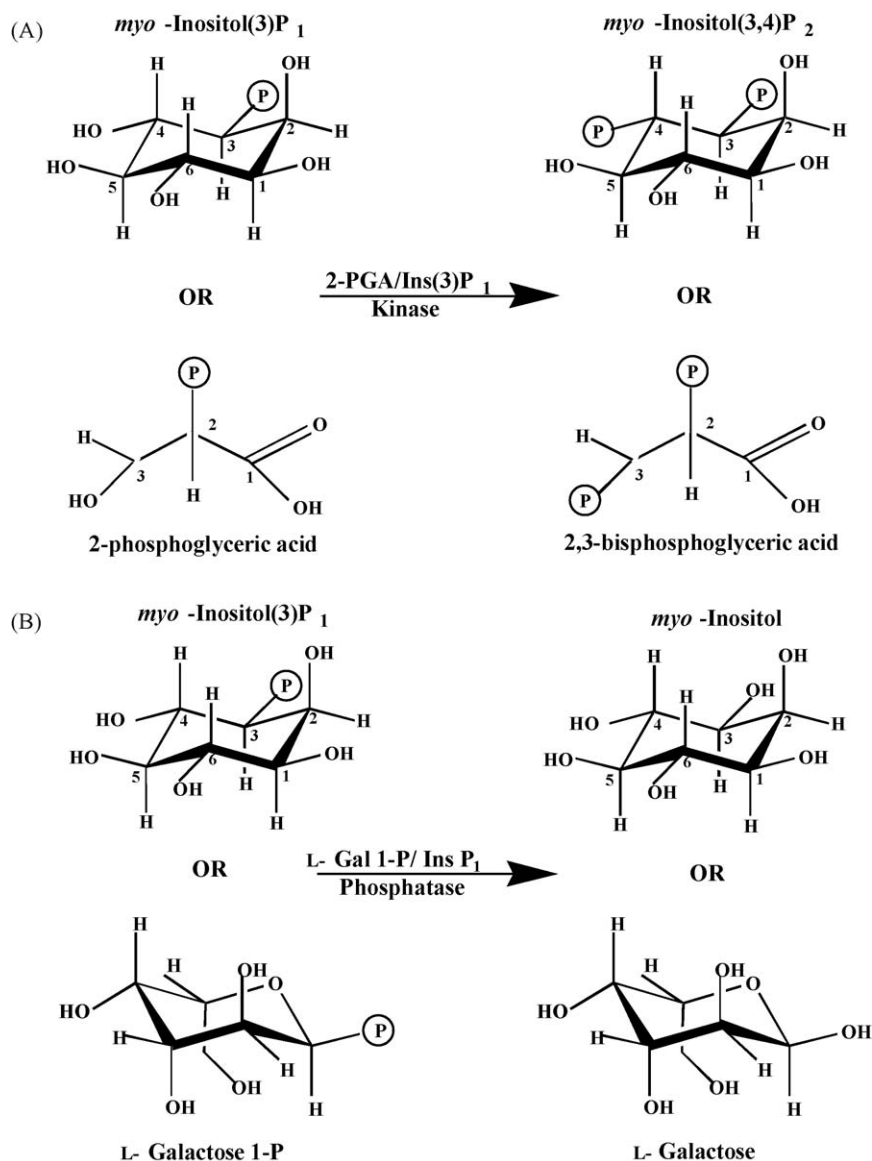
Fig. 5. Potential dual functionality of proteins in the inositol phosphate pathways might function to integrate metabolic pathways. (A) A 2-PGA kinase might also function as an inositol monophosphate kinase, providing the "missing link" in the "lipid-independent" pathway to phytic acid and integrating glyceric acid phosphate/glycolysis with inositol phosphate metabolism. This protein may catalyze both the synthesis of an intermediate (Ins P_2) and the synthesis of 2,3-bis-PGA that protects Ins P_2 from breakdown. (B) An inositol monophosphatase might also function as a galactose 1-P phosphatase, integrating inositol/inositol phosphate and perhaps both D- and L-galactose 1-P metabolism.

yield reductions are much more dramatic in non-irrigated (stressful) versus irrigated (less stressful) environments. It is likely that this reduced stress tolerance is due to perturbed Ins phosphate metabolism in vegetative tissues. Of the four barley *lpa* genotypes tested thus far, the best performer in both non-stressful and stressful environments was *lpa*1-1, a mutation that perturbs phytic acid accumulation in a seed-tissue-specific manner [85]. Phytic acid accumulation is reduced only in the aleurone layer of barley *lpa*1-1. Its embryo contains levels of phytic acid ≥ wild-type. In the other three barley *lpa* mutations both germ and aleurone phytic acid are reduced compared with wild-type. Barley *lpa*1 may be a target gene that can be manipulated via classical genetics and "mutation breeding" to achieve the same sort of trait targeting seen as an advantage to bioengineering approaches. Barley *lpa*1 is also important for controlling seed total P and its distribution (Section 5.1).

3.3. Downstream impacts on integrated metabolic pathways

The studies of rice *lpa*1 [73] and Ins/2,3-bis-PGA metabolism in *Dictyostelium* [74] and mammalian cells [76] indicate a linkage between Ins phosphate and glycolytic pathways. Several other lines of research indicate a linkage between Ins phosphate and carbon metabolism via the cellular galactose pool. First, Ins monophosphatases also function as D/L-galactose 1-P phosphatases [93–96]. This dual functionality may be important to the coordination of carbon flow in mammalian brain metabolism [93]. Mutations in a gene encoding an L-galactose 1-P phosphatase critical to ascorbic acid synthesis in *Arabidopsis* [94,95] also result in reduced cellular Ins levels [96]. Therefore two important branches of carbohydrate metabolism involving galactose and inositol may be linked and perhaps regulated at this metabolic junction (Fig. 5B). Plant genomes contain at least two lineages of sequences encoding proteins with potential galactose 1-P/Ins P_1

dual phosphatase activity [96]. Further studies are required to resolve their roles *in vivo*.

The pathways to phytic acid and the soluble seed oligosaccharides including the raffinosaccharide series, galactosyl derivatives of sucrose, and the galactosyl derivatives of cyclitols (in turn derivatives of inositol), all utilize inositol [43,97,98]. The raffinosaccharides include raffinose, stachyose and verbascose, and as a class are also referred to as the raffinose family oligosaccharides (RFOs) or the raffinose series oligosaccharides (RSOs). Their consumption causes flatulence and reduced raffinosaccharide level represents a breeding objective for improved end-use quality and consumer acceptance in soybeans and common beans [88]. The synthesis of the raffinosaccharides, cyclitols and galactosyl cyclitols are believed to be important to desiccation tolerance during seed maturation and therefore subsequent seed viability [97–99]. However, in the case of the raffinosaccharides, the exact relationship is not clear-cut. Studies that compared *Arabidopsis* or soybean genotypes that differ in endogenous levels of raffinosaccharides found little or no impact on seed viability, germination or emergence [100–102]. Mutations that produce the low-phytate phenotype via a block in Ins synthesis also result in low-raffinosaccharide, a desirable outcome [47]. The opposite is also the case. Mutations that block phytic acid synthesis downstream of Ins synthesis, for example via blocks in Ins or Ins phosphate kinases, reduce the sink for Ins which in some cases results in elevated Ins levels and elevated raffinosaccharides, a potentially undesirable outcome [88]. The potential importance of metabolic integration at the galactose 1-P/Ins P_1 pool is also indicated by the observation that both the pathways to raffinosaccharides and cell wall polysaccharides may utilize D-galactose 1-P for the generation of the galactose donor UDP-Gal, via the activity of a UDP-galactose/glucose pyrophosphorylase [103,104].

Impacts on germination and/or emergence may be common to mutations that perturb either Ins or phytic acid synthesis, and these may be due to downstream impacts on seed chemistry. Both the soybean LR-33 MIPS mutant and the soybean M153 ABC transporter mutant impact germination and seedling emergence, but much more so if the seed was produced in a tropical versus temperate nursery [105,106]. This "seed-source" effect indicates there is some difference between mutant and wild-type in seed maturation or chemistry that is critical to subsequent germination and emergence and that is more pronounced if the seed matures under tropical versus temperate conditions. Perhaps this seed-source effect is an outcome of reduced heat or desiccation tolerance during maturation that results in premature aging that in turn subsequently impacts germination and emergence. In the case of the soybean LR-33 MIPS mutant, a contributing factor may be the impact on seed raffinosaccharides, but the ABC transporter mutant is not known to impact raffinosaccharides. In the latter case the effect may reflect phytic acid's role in heavy metal chelation or as an antioxidant.

Phytic acid is an efficient chelator of iron ions and as such might function as an anti-oxidant, by removing free iron that catalyzes the Fenton reaction [12]. In the cereal grains phytic acid and most minerals accumulate in the germ, consisting of the diploid embryo and scutellum (the grass species' "monocotyledon"), and the triploid aleurone layer, which is genetically identical to but differentiated from the central, starchy endosperm [107]. At maturity the central endosperm contains little P, phytic acid or minerals. In maize, >80% of grain phytic acid is in the germ, with the remainder in the aleurone layer. In wheat, barley and rice the reverse is observed; >80% of grain phytic acid is in the aleurone layer, with the remainder in the germ. The localization in maize of phytic acid in the grain germ may result in a greater role, relative to other cereal crops, as an anti-oxidant important to seed viability. Germination rates are reduced in seeds homozygous for the maize

*lpa*1-241 allele, which conditions a 90% reduction in seed phytic acid [108]. This is even more pronounced upon "accelerated aging" and that this may be due to increased oxidative damage. Several indicators of oxidative damage were elevated in *lpa*1-241 kernels as compared with wild-type. They contained 50% more free or weakly bound radicals mainly concentrated in the embryo, higher levels of H_2O_2, and accelerated aging resulted in greater DNA damage and carbonylation of seed proteins.

The large and small changes in seed chemistry in *lpa* lines has significance to approaches to enhancing crop nutritional quality and to government regulations of transgenic organisms designed to safeguard the public from unexpected outcomes of new technologies. An example is the Canadian "Plant with Novel Traits" (PNT) regulations [109]. These regulations essentially require that any genetic, heritable change that substantially alters the chemistry of agricultural products, regardless of a given trait's source or how it is produced (transgenics, mutants, classical breeding, exotic germplasm), must be closely monitored and pass a rigorous permitting process. Low-phytate crops represented a test case for these regulations [109]. In Canada, barley is an important feed crop, and *lpa* barleys have been bred and registered as cultivars to provide one approach to managing P in animal production and to reducing the environmental impact of that production. These outcomes are highly desirable. However, seed P chemistry is greatly altered in *lpa* crops, and potentially undesirable outcomes may occur when using either classical genetics or genetic engineering to alter seed chemistry. Caution may be warranted in the use of these technologies, but excessive caution and risk aversion may also hinder technological progress.

3.4. Downstream impacts on signal transduction

Recent studies illustrate the central importance of both Ins phosphate metabolism and phytic acid in vegetative plant function, signal transduction and stress response. Overexpression in *Arabidopsis* of a mammalian Type 1 Ins polyphosphate 5-phosphatase, activity of which breaks down the second messenger Ins(1,4,5)P_3, results in enhanced drought tolerance [110]. The non-plant 5-phosphatase gene and protein are insensitive to endogenous regulatory systems, and their overexpression is speculated to dampen or modulate the Ins(1,4,5)P_3 signal in such a way as to enhance desiccation tolerance. A plant Ins polyphosphate 5-phosphatase protein contains a "WD40" repeat motif essential to stable protein-protein interactions, and one such interaction was shown to link Ins, sugar and stress signaling [111]. Phytic acid itself is incorporated in the core of a protein that functions in auxin signaling, the auxin receptor Transport Inhibitor Response 1 (TIR1) protein [112]. It is not known if phytic acid serves purely a structural function or both structural and regulatory functions. This is the second protein shown to contain phytic acid within its core. The human RNA editing enzyme, ADAR2, has phytic acid bound in its core and this is required for protein secondary structure and activity [113]. Roles have been ascribed for Ins phosphate and phytic acid in plant gravitropism and stomata biology [7,114]. Transcript profiling in developing seed homozygous for the barley low-phytate mutation *lpa*-M955, which blocks phytic acid synthesis by 90% throughout seed development, indicate reduced expression of genes important to the signaling pathways of the plant hormones cytokinin and ethylene, and to transport and synthetic processes important to cellular P, carbohydrate and cell wall synthesis (Section 5.1 [115]).

Vegetative and tuber tissues synthesize phytic acid and this might play a role in signal transduction important to disease resistance [116]. Potato leaf and tuber phytic acid levels were reduced via two transgenic approaches; antisense inhibition of MIPS expression (in this study referred to as "IPS" for *myo*-Inositol

Phosphate Synthase), or by introduction of an *Escherichia coli* polyphosphate kinase. The strategy behind the novel approach of introducing the bacterial polyphosphate kinase is that this enzyme uses ATP and catalyses the synthesis of variable length polyphosphate linear chains, not normally observed in higher plants, and thus depletes the cells of the ATP substrate for phytic acid synthesis. The resulting "low-phytic acid" potato plants were less resistant to the avirulent pathogen "potato virus Y" and to the virulent pathogen tobacco mosaic virus (TMV). The *Arabidopsis* genome contains three IPS/MIPS genes; *Atips1*, *Atips2*, and *Atips3* [116]. Mutant *atips2* plants were depleted in phytic acid and were hypersusceptible to four mosaic viruses, *Botrytis cinerea* and *Pseudomonas synrigae*. Mutant *atipk1* plants, which lack expression of an Ins polyphosphate 2-kinase, were also hypersusceptible. However, mutant *atips1* plants, while having reduced phytic acid, had wild-type levels of diseases resistance. Therefore it might not be the ability of tissues to synthesize phytic acid *per se* but rather a particular sub-cellular pool that is important for pathogen resistance [116]. The main flaw in this interpretation is the assumption that of all the downstream metabolic changes due to disruption of MIPS expression and reduced Ins synthesis, it is the reduced phytic acid or perturbed Ins phosphate metabolism that is important to disease resistance. In light of the many other pathways that utilize Ins, evidence for this specific causal relationship appears limited.

4. Breeding versus engineering high-yielding low-phytate crops

The results described above illustrate the many challenges to breeding a high-yielding low-phytate crop. Despite the interest in the low-phytate trait, to date there has only been one report [117] of selection within low-phytate germplasm for yield or performance. Such selection would combine allelic variants favorable for yield and performance specifically important in low-phytate genetic background. It would identify "modifiers" of the negative impacts of the low-phytate trait, which would overcome much of its associated agronomic problems. The performance of soybean low-phytate lines was enhanced by backcrossing followed by selection for seedling emergence [117].

Perhaps the simplest, low-tech approach to mitigating the negative effects of the low-phytate trait on plant or seed performance and stress-tolerance would be to utilize classical breeding with recessive alleles of genes encoding functions specific to phytic acid synthesis in seeds, those that have little role in vegetative Ins phosphate metabolism. Examples of such genes and functions include maize *lpa*3, which encodes an Ins kinase that displays embryo-specific expression [57], or genes like barley *lpa*1, whose expression appears to be seed aleurone-specific [85]. Probably mutations in these genes do no greatly impact vegetative, "house-keeping" Ins or Ins phosphate metabolism, and they may prove ideal for breeding low-phytate crops. However, they do impact seed chemistry and therefore could still have unforeseen consequences that impact seed quality, crop yield or performance.

The maize *lpa*1-ABC transporter has provided a good model for phytate engineering strategies [25]. Embryo-targeted suppression of *lpa*1expression produced the low-phytate trait in seed, but mitigated negative impacts on vegetative processes and yield. An alternative and potentially very powerful approach to problems associated with seed phytate is in engineering seed to accumulate high levels of phytase. The strategies utilized vary greatly. To enhance the bioavailability of iron in maize grain destined for human consumption, co-expression of a fungal phytase gene and a ferritin gene (for enhanced iron storage) was targeted to the maize endosperm [118]. The goal was to achieve high levels of active phytase in mature grains and therefore in foods prepared from the grain, not to engineer breakdown of phytates during seed development. The advantage of such an approach is that mature seed chemistry is largely unaltered, greatly reducing the risk for unanticipated negative impacts on plant or seed function, chemistry or quality. A heat-stable phytase was engineered for endosperm-targeted expression in wheat, since thermotolerant phytases better retain activity following food or feed preparation [119].

An alternative to these approaches is to engineer targeting of an active phytase that will both block phytic acid accumulation during seed development and accumulate as an active phytase in the mature seed. Engineering cytoplasm-specific expression of an *E. coli appA*-encoded phytase in developing soybean seeds achieved large reductions in phytate and also resulted in accumulations of an active phytase [24]. Such seeds had high-available phosphorus/low-phytate phosphorus, and the added advantage of providing an active phytase that upon ingestion breaks down phytates from other feed components. Initial studies indicated little effect on seed germination and yield, but confirmation via follow-up studies are required, such as stress–response testing. The potential problem with this approach is that seed chemistry is greatly altered, and this may have unanticipated consequences as described above.

5. Future directions

5.1. Seed total phosphorus

Most research addressing the biology of P in plants has focused on understanding and enhancing the ability of plants to take up soil P. Due to the prevalence of P-deficient production environments in the developing world, improved plant utilization of soil P is an important goal for international agriculture [120]. Nearly all this work has been with the model system *Arabidopsis*. Despite the large number of studies addressing P uptake and translocation, essentially none of the studies included assays of seed total P. Most molecular biology research in this area has addressed P uptake by roots, and in response to soil P deficiency, such as research addressing the "P starvation response" [121]. Despite the lack of focus or interest in seed total P, studies of plant P acquisition and P transport do suggest targets that might prove useful in engineering seed P levels. For example, differential gene expression patterns indicate that individual members of the *Pht*1 multigene family of P transporters might have a role specific to remobilization of P from vegetative tissues to the developing seed [122–124].

Only a few studies to date have directly addressed the genetics of seed total P. Two independent studies mapped quantitative trait loci (QTLs) that accounted for observed variation in seed total P [125,126]. Both studies identified a major QTL that accounted for a substantial amount of variation in both plant and seed P. A vacuolar ATPase contained within the chromosomal segment represented by this QTL may function in P transport important to seed P (Fig. 4). Additional *Arabidopsis* seed total P QTLs identified three *Pht*1 genes as candidates possibly responsible for the observed variation [126]. These studies begin the process of identifying potential targets for engineering seed total P.

Many lines of evidence suggest that the Ins phosphate signal transduction pathway plays an important role in P sensing and signaling in eukaryotes [1]. In *Saccharomyces* the acquisition of P and nitrogen, and the regulation of cellular Ins and P metabolic pools, are tightly integrated at the level of transcriptional regulation [127–131]. The transcriptional apparatus involved impacts chromatin remodeling and includes both P- and Ins-sensitive transcription factors [127]. The yeast homolog of the *Arabidopsis Ipk2* gene (perturbation of which results in the low-phytate trait in *Arabidopsis* [22]) is bifunctional in that the protein itself functions as a transcription factor responding to nitrogen

starvation [128]. The Ins phosphates produced by the *Ipk2* Ins polyphosphate 3-/6-kinase (multikinase) function as signaling ligands essential for the transcriptional regulation important to both Ins and P sensing [129,130]. Finally, Ins polyphosphates containing diphosphate moieties also have an essential function as ligands important to the regulation of gene expression in response to P starvation (Section 5.2 [131]).

This same level of integration of P, Ins and nitrogen response has not been reported in plant systems. Still, three studies indicate a role for Ins phosphates in both plant and seed P sensing/signaling. Mutations in the *Arabidopsis Ipk1* gene, encoding an Ins polyphosphate 2-kinase, both blocks seed phytic acid synthesis and also perturbs the ability of plants to sense and regulate vegetative P level, resulting in luxury uptake of P and vegetative P toxicity [22]. Since elevated plant P often results in elevated seed P, and since *Arabidopsis ipk1* mutants block phytic acid synthesis, they might produce seed with a "low-phytic acid/high-total P" phenotype, similar to one of the seed P phenotypes illustrated in Fig. 6. However, while seed P chemistry (Ins phosphate and inorganic P) was analyzed in this study [22], seed total P was not reported. In contrast, mutation of the barley *lpa*1 gene results in a shift in the distribution of total P in the seed (elevated in the germ and reduced in the endosperm/aleurone) and a 15% ($\pm$5%) reduction in seed total P [85]. Thus plant and seed P sensing appears perturbed in both the *Arabidopsis ipk1* and barley *lpa*1 mutants, altering P uptake and distribution in both cases, but apparently in opposite ways. Barley cultivars that have adequate yield have been bred and registered using barley *lpa*1-1 [132]. Thus barley *lpa*1 represents a proof-of-principle that single-gene allelic variants can be identified that specifically result in reduced seed total P, while having relatively little impact on seed and plant performance.

Transcript profiling during seed development of the barley *lpa*-M955 mutant indicates a role for Ins phosphates and phytic acid in hormonal signaling and illustrated the coordination of functions important to P and carbon metabolism [115]. Seed phytic acid synthesis is almost abolished in developing barley *lpa*-M955 and nearly all P over and above a minimal level necessary for basal metabolism accumulates as inorganic P. Using a microarray that

assays expression of over 14,000 genes, differential gene expression ($\geq$2-fold difference between *lpa*-M955 and wild-type isolines) was observed for only 38 genes. Of these, only two were up-regulated. Of the remaining 36 highly down-regulated genes, two encoded functions critical to the molecular mechanism underlying cytokinin signal transduction, the "histidine-aspartate phosphor-elay pathway" [133,134]. The His-Asp P-relay pathway detects a cytokinin signal at the exterior of the cell wall via a protein kinase receptor. This protein transduces the signal to the cell interior where the signal is first transferred via a "histidine-containing phosphotransfer protein" (or via a similar domain of a hybrid protein) to a "response regulator", which then enters the nucleus to effect change in gene expression. The two genes down-regulated in *lpa*-M955 seed encoded a histidine-containing phosphotransfer protein and a response regulator. Thus the block in phytic acid synthesis in seeds has a large impact on the expression of genes important to cytokinin signal transduction. The importance of cytokinin signaling to nitrogen, P and sulfur acquisition and regulation is well known [135]. The small number of genes whose expression was clearly down-regulated in *lpa*-M955 as compared with wild-type also included those encoding an alpha-amylase inhibitor, a brittle-1 protein, an ethylene-response protein, a pyrophosphatase and sucrose synthase 2 [115]. These results clearly indicate that there are coordinated P and carbon sensing/signaling processes in the developing seed that parallel those in the parental plant, and that involve Ins phosphates and phytic acid.

The lack of focus or interest in the genetics of seed total P probably indicates that genetic screens for mutations and allelic variants that specifically impact seed total P would yield new resources for study and new targets for bioengineering. Two types of genetic screens are currently being conducted. In Screen 1 (Fig. 6, right) a high-throughput inorganic P assay is used to screen progeny obtained following the chemical mutagenesis of an *lpa* genotype for any mutation that alters the high-inorganic P phenotype typical of the parental *lpa* genotype. The rationale is that as inorganic P represents that bulk of seed total P in an *lpa* genotype, mutations that increase or decrease seed total P will result in readily detectable increases or decreases in inorganic P. The hypothetical "low-phytic acid:low-total P" mutant illustrated in Fig. 6 might turn out to have the ideal seed P amount and chemistry for nearly all end-uses as it has both low-total P and "high-inorganic P/high-available P", as compared with wild-type. In Screen 2 (Fig. 6, left), a tissue total P assay is used to screen seed from chemically mutagenized populations for mutations that alter seed total P. The assay is directly for changes in total P, not indirectly via changes in the inorganic P component of total P, as in Screen 1. In Fig. 6, left, Screen 2 is illustrated using a wild-type, normal phytic acid germplasm. However, since the tissue total P assay used in this screen is not dependent on the high-inorganic P characteristic of *lpa* lines, any germplasm or population of any genetic background can be screened, including *lpa* populations, transposon insertion populations, or germplasm collections. Although absolute levels of seed phytic acid are altered in the hypothetical mutants illustrated in Fig. 6, left, they have "normal phytic acid" in the sense that the ability of seeds to synthesize phytic acid is not perturbed, and the proportion of total P found as phytic acid P is not altered. Regardless of the screening method used, it is likely that these genetic screens will yield new resources for studying the biology of seed total P and for breeding high- or low-total P lines.

5.2. Diphosphate and triphosphate-containing inositol polyphosphates

One of the more currently active aspects of Ins phosphate research in non-plant eukaryotic systems addresses the class of Ins
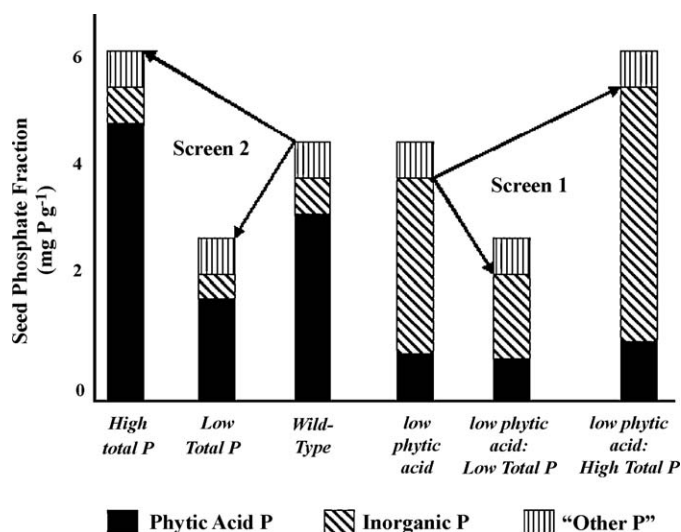

Fig. 6. Two types of screens for altered seed total P. Screen No. 1 uses an assay for inorganic P and is for mutations that alter the seed inorganic P phenotype of *low-phytic acid* mutations. Screen No. 2 uses a seed total P assay and will be used with both wild-type and lpa populations. On the right is a parental *low-phytic acid* line and two hypothetical second-site mutations derived from it that either increase or decrease seed total P in a *low-phytic acid* background. On the left is a "normal phytic acid" wild-type parental line and two hypothetical mutations that either increase or decrease seed total P in a "normal phytic acid" background.
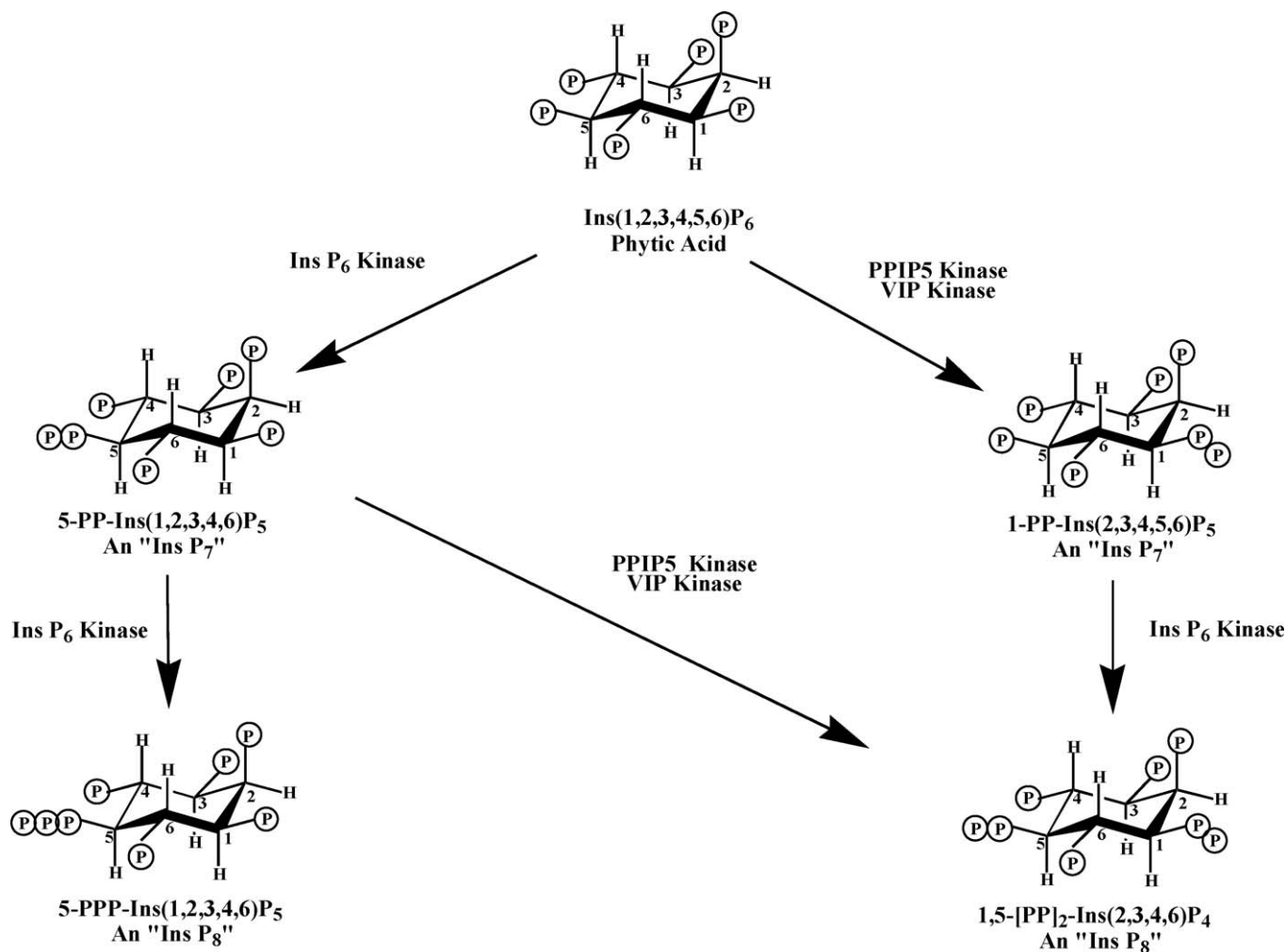
Fig. 7. The synthesis of inositol polyphosphates containing biphosphate (PP) or triphosphate (PPP) moieties, starting with phytic acid as the initial substrate. These compounds and pathways have been well documented in non-plant eukaryotic systems, but have not yet been studied in detail in higher plant systems.

phosphates that contain diphosphate (PP-Ins phosphates or Ins pyrophosphates) or triphosphate moieties (PPP-Ins phosphates; Fig. 7 [136]). These compounds can contain seven or more moles of P per mole of Ins, as compared with phytic acid's six moles of P per mole of Ins. PP- and PPP-Ins phosphates may have multiple functions ranging from P sensing, to actin cytoskeleton dynamics, apoptosis, vesicle trafficking, DNA repair, protein phosphorylation, and ATP regeneration [131,136,137]. In mammalian cells these molecules undergo "exceptionally high rates of metabolic turnover" via coupled kinase/phosphatase activities [138]. Mammalian cells therefore expend a lot of energy and resources on their metabolism, one indication of their presumptive importance. They may serve as "metabolic messengers", coupling signaling pathways to the energetic status of a cell [138]. Studies of the shifts in gene expression in response to abiotic stress have led to a consensus that abiotic stress impacts the energy status of tissues and that the highly conserved energy sensor protein kinases, the yeast SNF1 (sucrose non-fermenting 1) and the plant SnRK1 (SNR1-related kinase 1), function to sense this status and regulate gene expression in response [139,140]. Ins phosphates including the PP-Ins phosphates probably play an intimate role as signals/ligands in SNF1/SnRK1-mediated regulation, which is also central to cellular P status response [131]. There has little work on PP- and PPP-Ins phosphates in plant systems, other than three initial reports of their chromatographic detection [141–143].

Research to date has identified two types of enzymes that catalyze the synthesis of PP- or PPP-Ins phosphates. An "Ins P_6 kinase" phosphorylates the "5" position either once to form 5-PP-Ins(1,2,3,4,6)P_5, a Ins phosphate containing seven moles of P per mole of Ins, with a single phosphate ester at the "1", "2", "3", "4" and "6" positions, and a diphosphate or pyrophosphate at the "5" position (Fig. 7). The same enzyme may then add an additional phosphate at the "5" position to form a PPP- or triphosphate moiety [144]. Ins P_6 kinase is a member of the same superfamily of Ins polyphosphate kinases that includes the Ins polyphosphate 3-/6-kinases (multikinases) including the *Arabidopsis Ipk2β* shown to be important to phytic acid synthesis in seeds [22]. The "diphosphoinositol pentakisphosphate kinase" (PPIP5 kinase) phosphorylates the "1" or "3" position of phytic acid, for example to form a 1-PP-Ins(2,3,4,5,6)P_5, an Ins phosphate containing seven moles of P per mole of Ins, with the diphosphate at the "1" position (Fig. 7) [144,145]. PPIP5K is encoded by the yeast *VIP* gene and plant genomes contain well conserved homologs [80]. Finally, these two types of kinases can act together to synthesize Ins polyphosphates with diphosphates at both the "1" or "3" position and the "5" position [145].

The function(s) of PP-Ins and PPP-Ins phosphates in plants is unknown at present. Many components of the Ins phosphate pathways are well conserved across eukaryotes, therefore PP-Ins phosphate metabolism may be important to P-sensing and to

abiotic stress sensing and signaling in plants. In a parallel to one role of phytic acid and PP-Ins phosphates in the cellular slime molds *Dictyostelium* and *Polysphondylium pallidum* [5], these compounds may play an as yet unrecognized role in phytic acid synthesis and storage in higher plant seeds. At low cell densities the slime molds exist in a "vegetative" stage as solitary amoeba, and upon starvation either form multicellular aggregates that differentiate into spores supported on stalks (*Dictyostelium*), or form microcysts (*Polysphondylium*), both of which subsequently germinate to release solitary amoeba. During the amoeba cellular stage, at relatively low cellular densities, the levels of phytic acid and PP-Ins phosphates remain low, from $\sim$10 μM for PP-Ins phosphates up to $\sim$250 μM for phytic acid. At higher cell densities phytic acid increases to $\sim$650 μM and the PP-Ins phosphates to $\sim$250 μM. Upon starvation and spore formation both phytic acid and PP-Ins phosphates can increase up to $\sim$2.0 mM. During subsequent germination cellular Ins P_6 and PP-Ins phosphate levels return to basal, "vegetative" levels. This pattern of accumulation during development of tissue and organs that acquire nutrient stores and enter resting stages, and subsequent breakdown during germination, is very similar to that observed for phytic acid in tissues and organs involved in reproduction of higher plants.

A third role for the PP-Ins and PPP-Ins phosphates of potential importance to seed biology is as a "P-bond energy" reserve. The PPIP5 kinase can also act in the reverse direction, using bis-PP-InsP$_4$ as a phosphate donor in the regeneration of ATP from ADP, "an indication of the high-phosphoryl group transfer potential of bis-PP-InsP$_4$" [137]. This is very reminiscent of the much earlier hypothesis [146] that phytic acid could serve as a donor for the regeneration of ATP during seed development, an endosperm tissue version of the "phosphagen" creatine phosphate. This early hypothesis was dismissed at first due to thermodynamic considerations; the standard free energy of hydrolysis of single phosphomonoesters such as those found in phytic acid, perhaps 3–4 kcal mol^{-1}, is not sufficient to drive ADP $\rightarrow$ ATP, which requires $\sim$7 or more kcal mol^{-1} (numbers are rough estimates). However, in this case kinetic considerations may be more important than purely thermodynamic considerations [147]. If for example the relative concentrations of phytic acid and ADP are high, and ATP low, and in the presence of a catalyst such as an enzyme with appropriate activity, then phytic acid might serve as P donor. This is exactly the case of the cereal grain aleurone layer during imbibition and early germination. At maturity the aleurone layer really is a "phytate layer", where phytic acid represents $\sim$20% of its dry weight [1]. Upon imbibition, if water accounts for 80% of the aleurone layer's fresh weight, the concentration of phytic acid would be 50 mM. Two studies [148,149] have demonstrated that Ins polyphosphate 2-kinase, the enzyme encoded by the *Arabidopsis Ipk1* gene [22] could, under the right substrate concentrations (high-phytic acid, high ADP), catalyze the reverse reaction and generate ATP from ADP. In summary it appears clear that the highly phosphorylated inositols, including phytic acid and the PP-Ins and/or PPP-Ins phosphates, together may function as "phosphagens", under the right cellular circumstances. This could be critically important to ATP regeneration during the early stages of seed germination, when the lack of mitochondrial membrane integrity, required for chemiosmotic mechanisms, prevents oxidative phosphorylation. In light of their potential roles in seed phytic acid metabolism, plant and seed P sensing and total P levels, seed germination and stress biology, clearly more research is needed in plant science that addresses PP-Ins and PPP-Ins phosphate biology.

6. Conclusion

Areas of Ins phosphate and phytic acid biology in plants certainly remain that are unexplored or not fully understood. The clearest example is PP-Ins and PPP-Ins phosphate biology. In addition, more work is needed to achieve a greater understanding of the role of Ins phosphates and phytic acid in the integration and functioning of metabolic and hormonal signaling pathways. However, a great deal of progress has been made in the genetics and genetic engineering of the low-phytate and high-phytase traits in seed crops. In the past it had been common to read that the pathways to phytic acid were not well understood. That is no longer true. Many if not most of the genes and proteins involved in phytic acid metabolism have been identified. Substantial resources now are available to engineer high levels of active phytase protein in seeds crops. Therefore a variety of technologies and approaches are available to engineer the low-phytate and/or high-phytase trait, possibly soon leading to high-yielding, stress-tolerant, consistently performing low-phytate or high-phytase seed crops. However, careful studies are still needed to test the performance characteristics, safety and utility of low-phytate versus high-phytase crops. Until such studies are completed, the relative merits of these alternative approaches to the phytic acid problem cannot be determined. In contrast, there has been little progress in the genetics and engineering of seed total P. Thus there is outstanding opportunity for new research and development aimed at developing either "high-seed P" or "low-seed P" crops, or "low-phytate/low-total P" crops, the latter potentially useful in enhancing the sustainability and decreasing the environmental impact of agricultural production.

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